AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

In Vivo Gentamicin Susceptibility Test for Prevention of Bacterial Biofilms in Bone Tissue and on Implants

Louise Kruse Jensen,[a] Thomas Bjarnsholt,[b,c] Kasper N. Kragh,[b] Bent Aalbæk,[a] Nicole Lind Henriksen,[a] Sophie Amalie Blirup,[a] Karen Pankoke,[a] Andreas Petersen,[d] Henrik Elvang Jensen[a]

[a]Department of Veterinary and Animal Sciences, University of Copenhagen, Copenhagen, Denmark
[b]Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark
[c]Department of Clinical Microbiology, Copenhagen University Hospital, Copenhagen, Denmark
[d]Statens Serum Institut, Copenhagen, Denmark

ABSTRACT The objective of this study was to set up an *in vivo* gentamicin susceptibility test for biofilm prevention in bone tissue and on implants. Twenty-five pigs were allocated to six groups. Pigs in group A ($n = 6$) were inoculated with saline. Pigs in groups B ($n = 6$), C ($n = 3$), D ($n = 3$), E ($n = 3$), and F ($n = 4$) were inoculated with 10 μl saline containing 10^4 CFU of *Staphylococcus aureus*. Different concentrations based on the MIC of gentamicin for the specific strain were added to the 10-μl inoculum for groups C (160× MIC), D (1,600× MIC), E (16,000× MIC), and F (160,000× MIC). The inocula were injected into a predrilled tibial implant cavity, followed by insertion of a steel implant (2 by 15 mm). The pigs were euthanized after 5 days. *In vitro*, all the doses used were found to be bactericidal after up to 6 h. All implant cavities of pigs inoculated with bacteria and bacteria plus 160× MIC or 1,600× MIC of gentamicin were positive for *S. aureus*. In animals in each of groups E (16,000× MIC) and F (160,000× MIC), 2/3 and 1/4 of the implant cavities were *S. aureus* positive, respectively. By grouping groups C and D (<10,000× MIC) and groups E and F (>10,000× MIC), a significant decrease in the number of implant-attached bacteria was seen only between the high-MIC-value group and group B. Histologically, it was demonstrated that 1,600×, 16,000×, and 160,000× MIC resulted in a peri-implant tissue reaction comparable to that in saline-inoculated animals. *In vivo*, the antimicrobial tolerance of the inoculated planktonic bacteria was increased by *in vivo*-specific factors of acute inflammation. This resulted in bacterial aggregation and biofilm formation, which further increased the gentamicin tolerance. Thus, susceptibility patterns *in vitro* might not reflect the actual *in vivo* susceptibility locally within a developing infectious area.

KEYWORDS aminoglycosides, animal models, biofilms, susceptibility testing

Bacterial biofilms are involved in many chronic infections, such as pneumonia in patients with cystic fibrosis, catheter-associated infections, prosthetic joint infections, and osteomyelitis (1). Once embedded in a biofilm, the bacteria profoundly change their metabolism and the tolerance toward antimicrobials is increased, which enables persistence of the infection (1). Several parameters have been developed to quantify the antimicrobial activity against free planktonic bacteria and bacteria living in biofilms (2). In line with the well-known MIC, a minimal biofilm inhibitory concentration (MBIC), a biofilm bactericidal concentration (BBC), and a minimal biofilm eradication concentration (MBEC) have been defined (3, 4, 5, 6). It has been demonstrated that the antimicrobial concentrations needed to eradicate already established biofilms are higher than the concentrations required to kill the same bacterial clone cultured planktonically (7, 8). Thus, in a study based on 53 *Staphylococcus aureus* isolates from

Citation Jensen LK, Bjarnsholt T, Kragh KN, Aalbæk B, Henriksen NL, Blirup SA, Pankoke K, Petersen A, Jensen HE. 2019. *In vivo* gentamicin susceptibility test for prevention of bacterial biofilms in bone tissue and on implants. Antimicrob Agents Chemother 63:e01889-18. https://doi.org/10.1128/AAC.01889-18.

Address correspondence to Louise Kruse Jensen, louise-k@sund.ku.dk.

Received 25 September 2018
Returned for modification 13 October 2018
Accepted 12 November 2018
Accepted manuscript posted online 19 November 2018
Published 29 January 2019

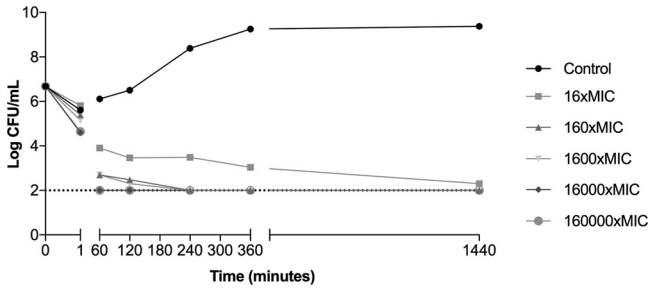

FIG 1 *In-vitro* time-kill study. The results represent the viable count (in number of CFU per milliliter) of *Staphylococcus aureus* strain S54F9 in the time-kill experiment with different gentamicin concentrations (16, 160, 1,600, 16,000, and 160,000 times the MIC). On the *x* axis, 0 represents the number of CFU just before gentamicin exposure. The dotted line at a value of 2 CFU/ml on the *y* axis represents the detection limit.

diabetic bone infections, the MBIC values were increased by a factor of 1,000 compared to the MIC (9). The biofilm prevention concentration (BPC) has been described as a modification of the MBIC, with the aim of reducing the bacterial density to prevent biofilm formation (5, 10).

The principles behind MBIC, BBC, MBEC, and BPC are that they all explore the activity of antimicrobials *in vitro*. However, it has recently been pointed out that *in vitro* biofilm susceptibility tests often give results that poorly mimic the antimicrobial activity against *in vivo* biofilms (11, 12). Therefore, the aim of the present study was to set up an *in vivo* single-dose antimicrobial susceptibility test for biofilm prevention. The study was based on adding different concentrations of gentamicin to the inoculum in a discriminative porcine model of implant-associated osteomyelitis (13).

RESULTS

MIC of gentamicin. The MIC value of gentamicin (100 mg/ml; Genta-Equine) for *S. aureus* strain S54F9 was determined to be 0.25 mg/liter.

Time-kill curve study of gentamicin. The bactericidal activity was concentration dependent (Fig. 1). After 24 h, only the control and the sample containing 16 times the MIC value of gentamicin had a viable bacterial count above the detection limit (10^2 CFU/ml). For the high MIC values (16,000× and 160,000× MIC), the detection limit was encountered within 2 h, and for the low MIC values (160× and 1,600× MIC), the limit was encountered after 4 h. The time-kill curves demonstrated that mixing of bacteria and gentamicin for 60 s prior to inoculation in the porcine models did not eliminate the inoculum; i.e., bacteria were present in all samples 1 min after exposure to gentamicin.

Microbiology and sonication of implants. The results of bacterial detection with swabs, sonication (implant biofilm), and immunohistochemistry (IHC) (tissue biofilm) can be found in Table 1. All control animals in group A were found to be sterile, and the group B animals (bacteria only) were positive in at least two of the three analyses. In general, no bactericidal effect was seen for gentamicin at 160× and 1,600× MIC (Table 1). In contrast, an effect was seen with gentamicin concentrations of 16,000× and 160,000× MIC (Table 1). All positive swabs were confirmed to contain *S. aureus* bacteria of *spa* type t1333 (identical to the *spa* type used for inoculation). A statistically significant decrease in the number of implant-attached bacteria ($P = 0.001$) was seen only between the high-MIC-value group (>10,000× MIC [groups E and F]) and group B (bacteria only) (Fig. 2A).

Pathology. Macroscopic signs of infection were not seen in groups A, E (bacteria plus 16,000× MIC of gentamicin), and F (bacteria plus 160,000× MIC) (Fig. 3A), except in one group F animal, which had pus in the implant cavity. Pus was found in the implant cavity of all group B (bacteria only), C (bacteria plus 160× MIC), and D (bacteria plus 1,600× MIC) animals (Fig. 3B and C). The implant cavity was irregular due to bone necrosis in group B and C animals (Fig. 3B and C). In all control animals belonging to group A, the peri-implant pathological bone area (PIBA) consisted of an interrupted

TABLE 1 Overview of study groups and detection of bacteria postmortem

Group	Animal no.	Inoculum	Detection of bacteria in implant cavity (swab)	Detection of biofilm in:	
				Implant (sonication)	Tissue around implant cavity (IHC[a])
A	A1	Saline	No		No
	A2	Saline	No		No
	A3	Saline	No		No
	A4	Saline	No	No	No
	A5	Saline	No	No	No
	A6	Saline	No	No	No
B	B1	*S. aureus*	Yes (inoculated *spa* type)	Yes	Yes
	B2	*S. aureus*	Yes (inoculated *spa* type)	Yes	No
	B3	*S. aureus*	Yes (inoculated *spa* type)	Yes	Yes
	B4	*S. aureus*	Yes (inoculated *spa* type)	Yes	Yes
	B5	*S. aureus*	Yes (inoculated *spa* type)	Yes	Yes
	B6	*S. aureus*	Yes (inoculated *spa* type)	Yes	Yes
C	C1	*S. aureus* + 160× MIC (40 mg/liter)	Yes (inoculated *spa* type)	Yes	Yes
	C2	*S. aureus* + 160× MIC	Yes (inoculated *spa* type)	Yes	No
	C3	*S. aureus* + 160× MIC	Yes (inoculated *spa* type)	Yes	Yes
D	D1	*S. aureus* + 1,600× MIC (400 mg/liter)	Contamination	Yes	No
	D2	*S. aureus* + 1,600× MIC	Yes (inoculated *spa* type)	Yes	No
	D3	*S. aureus* + 1,600× MIC	Yes (inoculated *spa* type)	No	No
E	E1	*S. aureus* + 16,000× MIC (4,000 mg/liter)	No	Yes	Yes
	E2	*S. aureus* + 16,000× MIC	No	No	No
	E3	*S. aureus* + 16,000× MIC	Yes (inoculated *spa* type)	Yes	No
F	F1	*S. aureus* + 160,000× MIC (40,000 mg/liter)	No	No	No
	F2	*S. aureus* + 160,000× MIC	Yes (inoculated *spa* type)	Yes	No
	F3	*S. aureus* + 160,000× MIC	No	No	No
	F4	*S. aureus* + 160,000× MIC	No	No	No

[a]IHC, immunohistochemistry for *S. aureus*.

thin layer of elongated fibroblasts lining compressed and osteonecrotic trabecular bone tissue. This layer was sporadically intermingled with single neutrophilic granulocytes. In contrast, PIBA of animals inoculated with bacteria only showed more osteonecrosis and had a massive infiltration of neutrophil granulocytes, macrophages, giant cells, proliferating fibroblasts, and active osteoclasts. For group C animals (160× MIC), PIBA morphology, measurements, and neutrophilic granulocyte (NG) counts were comparable to those in the pigs in group B (bacteria only) (Fig. 2B and C and Fig. 4A, C, and D). For animals in groups D (1,600× MIC), E (16,000× MIC), and F (160,000× MIC), PIBA morphology, measurements, and NG counts were comparable to those in the saline-inoculated control animals in group A (Fig. 2B and C and Fig. 4B).

DISCUSSION

The present study describes an *in vivo* antimicrobial susceptibility test of biofilm prevention in bone tissue and on implants. We found that 1,600 times the MIC value (or 400 mg/liter) of gentamicin was needed in the inoculum (10 μl with 10^4 CFU) in order to prevent bacterial attachment to bone implants in the porcine model of implant-associated osteomyelitis. However, looking only at the bone tissue, all concentrations above 40 mg/liter resulted in a tissue response comparable to that in the saline-inoculated animals. All the gentamicin doses used were 100% effective *in vitro* against the planktonic form of the *S. aureus* strain used, but once the planktonic bacteria were injected *in vivo*, they showed an increased gentamicin tolerance due to inflammation and the beginning of biofilm formation.

Previously, in an *in vitro* biofilm susceptibility study, the BPC values of different antimicrobials were only slightly higher than their MIC values against *Pseudomonas aeruginosa* (5). This was supported by another study, in which antibiotic-loaded bone

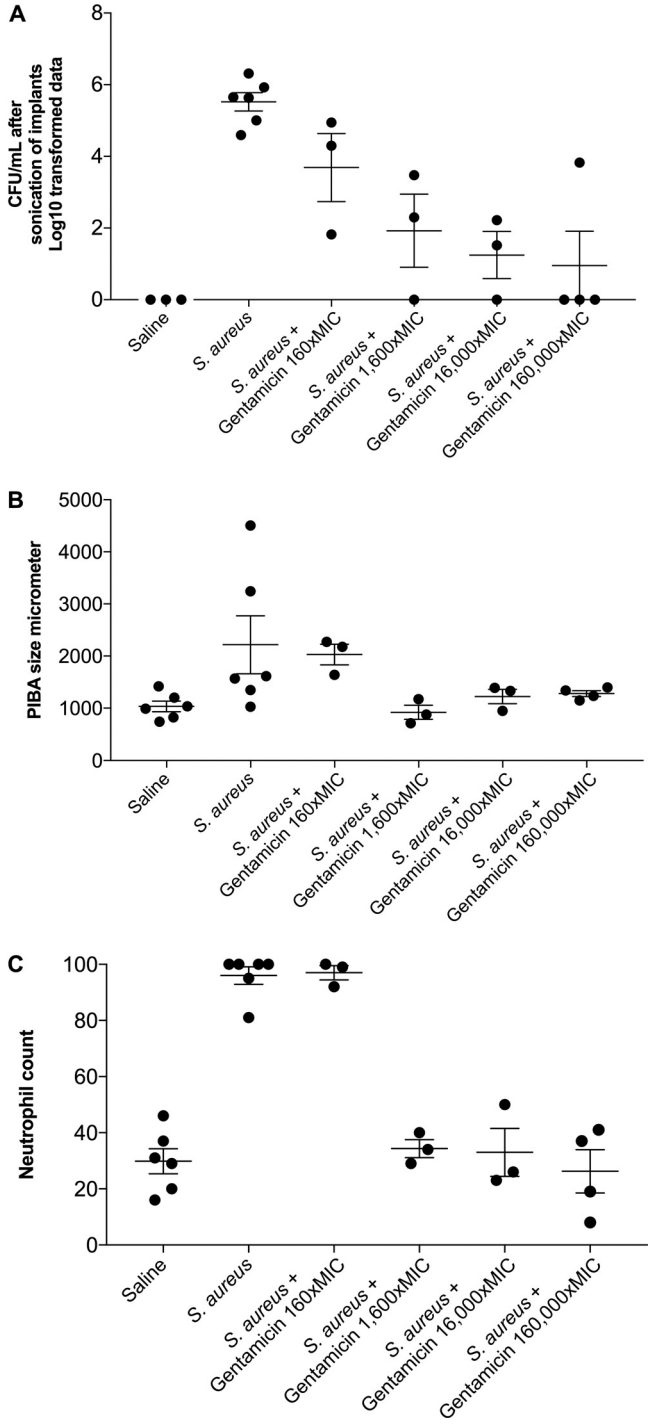

FIG 2 Results of sonication and histological registrations. (A) The number of bacteria attached to the implants was reduced with the increasing values of the MIC of gentamicin added to the inoculum. (B and C) The width of the peri-implant pathological bone area (PIBA) (B) and the count of neutrophilic granulocytes (C) were increased only in animals inoculated with bacteria and with bacteria plus 160× MIC gentamicin. Single values and the mean and standard error of the mean (SEM) are shown for each group.

beads were able to prevent local biofilm formation by planktonic *S. aureus* bacteria, while an established biofilm was untreatable (10). In contrast, another recently published *in vitro* study demonstrated that large amounts of gentamicin (171 to 1,260 mg/liter) released from a biphasic gentamicin-loaded calcium sulfate-hydroxyapatite bone

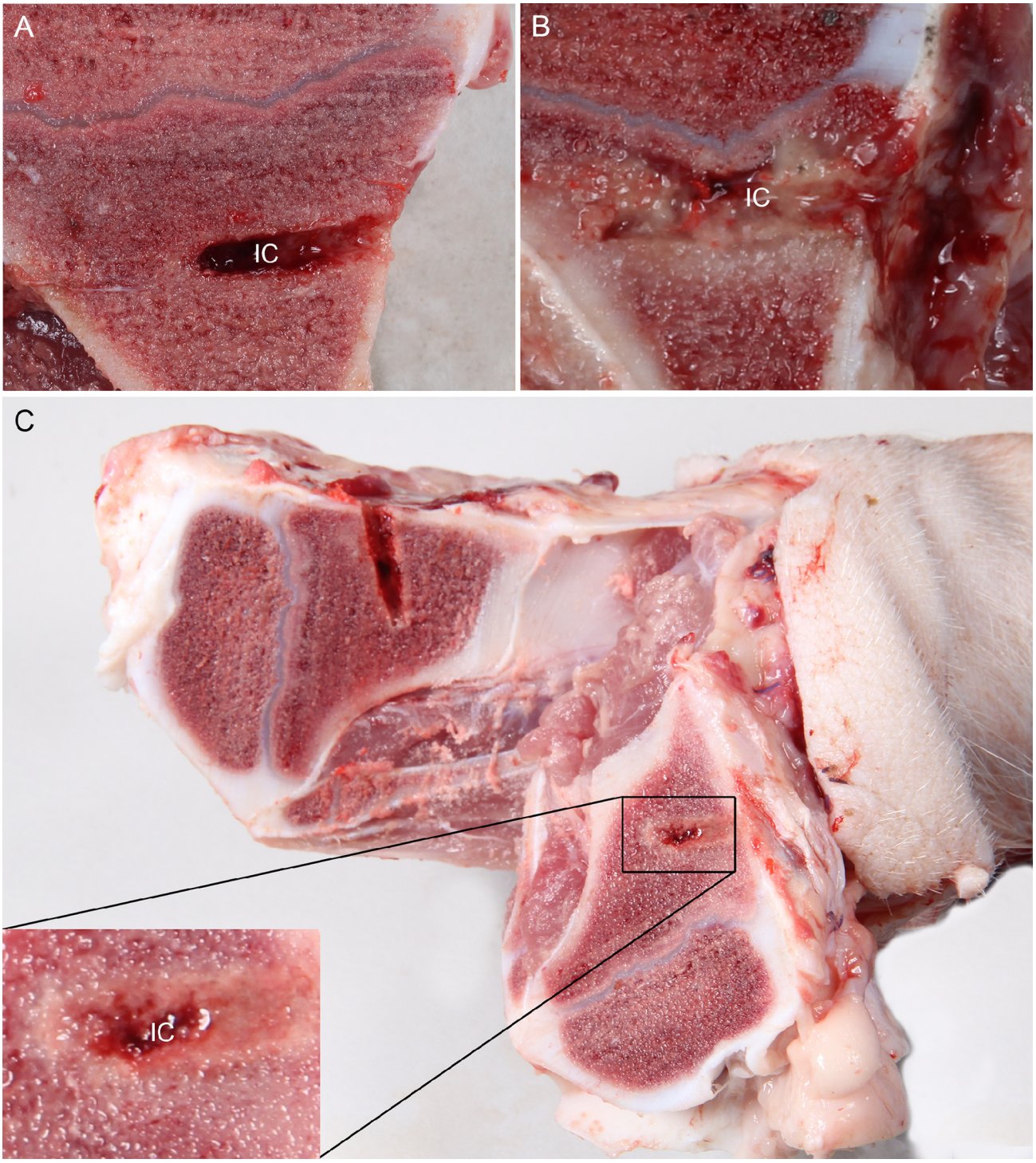

FIG 3 Macroscopic bone pathology. Three pigs were inoculated with *Staphylococcus aureus* and gentamicin at different MICs in the right tibia, followed by insertion of a steel implant. The pigs were euthanized 5 days after inoculation. The tibial bone was sagittally sectioned, and the implant was removed for demonstration of the implant cavity (IC). (A) Pig inoculated with bacteria and 160,000× MIC of gentamicin. No pus was visible, and the implant cavity is regular. (B) Pig inoculated with bacteria and 160× MIC gentamicin. Pus and bone destruction surround the implant cavity. (C) Pig inoculated with bacteria and 1,600× MIC of gentamicin. Although there was less pus than in the image in panel B, the bone contour of the implant cavity was still destroyed (inset).

graft substitute were needed to prevent and eradicate biofilms of Gram-positive bacteria (14). Our results also demonstrate that successful antimicrobial prevention of biofilms requires concentrations above the MIC. However, the effective *in vivo* concentration must be much higher than that previously demonstrated *in vitro* due to the

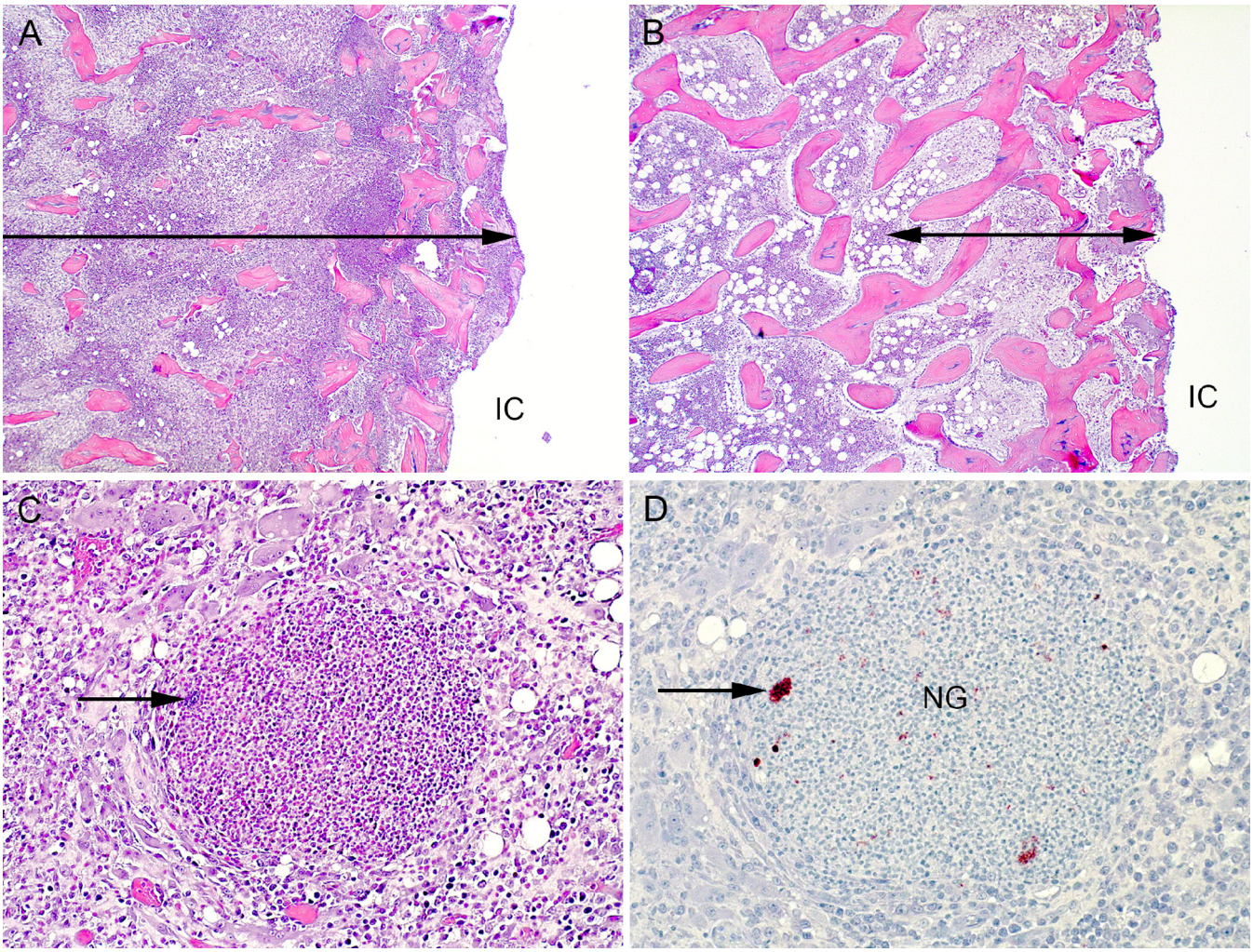

FIG 4 Microscopic bone pathology. Two were pigs inoculated with *Staphylococcus aureus* and different MICs of gentamicin in the right tibia, followed by insertion of a steel implant. The pigs were euthanized 5 days after inoculation. The tibial bone was sagittally sectioned, and the implant was removed for demonstration of the implant cavity (IC). (A) Pig inoculated with bacteria and 160× MIC of gentamicin. HE stain was used. The arrow indicates the extension of the peri-implant pathological bone area (PIBA). PIBA contains necrotic bone tissue and a massive infiltration of neutrophilic granulocytes and macrophages. (B) Pig inoculated with bacteria and 160,000× MIC of gentamicin. HE stain was used. PIBA is small (double arrow), and only a sparse inflammatory reaction was present. (C) Close-up of the image in panel A. A microabscess that contained small aggregates of bacteria (arrow) and that was surrounded by multinuclear giant cells is seen. (D) Within the microabscess, *S. aureus* was detected immunohistochemically. Red positive bacteria (arrow) can be seen among the neutrophilic granulocytes (NG).

impact of inflammation (7). Thus, susceptibility patterns *in vitro* might not reflect the actual *in vivo* susceptibility locally within a developing infectious area. Therefore, the present findings support the suggestion that it seems to be insufficient to evaluate the prophylactic concentration of antimicrobials for the prevention of biofilms based on *in vitro* assays (11).

The formation of a biofilm *in vitro* is not representative of the formation *in vivo* (15). *In vivo*, the tissue hosting a biofilm or surrounding a medical device covered with a biofilm impacts the biofilm oxygen supply, size (5 to 200 μm), and extracellular matrix composition (15). Moreover, the *in vivo* biofilm stimulates the adjacent tissue to produce a local inflammatory response. Within the present study, drilling of the bones resulted in an implant cavity surrounded by hemorrhage, bone debris, and thermic bone necrosis. Once injected into the implant cavity, the inoculum triggered acute inflammation with vasodilation and increased vascular permeability, allowing additional fluid and plasma proteins to pass into the implant cavity (16). These circumstances may have contributed to a dilution of the gentamicin and coating of the

implants with plasma proteins, like fibronectin. It has been demonstrated that *S. aureus* biofilm formation on artificial surfaces is initiated when microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) bind to plasma proteins embedded on the surface (17, 18). Therefore, in the pigs it seems that biofilm formation occurred right after inoculation due to bacterial attachment to MSCRAMMs on the implant and the necrotic bone tissue induced by drilling. Biofilm-associated antimicrobial tolerance develops in normally susceptible bacteria when they form biofilms due to the display of an altered phenotype (9). Moreover, the inflammatory response induced by the bacteria gives an acidic environment, and it has been demonstrated that a low pH increases the MICs of aminoglycosides for Gram-positive cocci (19). Gentamicin is a concentration-dependent bactericidal aminoglycoside, and it has been demonstrated *in vitro* that concentrations above 8 times the MIC do not result in significantly faster killing of bacteria (20). All the gentamicin doses used in the present study might have induced killing of the planktonic bacteria when added to the inoculum; however, once injected into the pigs, only the high gentamicin doses ($>$10,000$\times$ MIC) continued to have a sufficient bactericidal concentration, despite the inflammatory reaction and the beginning of biofilm development.

The observed effective gentamicin doses cannot be achieved in bone tissue with systemic administration. Comparable conclusions have been made from several *in vitro* studies of biofilm susceptibility (21). To meet the demand for high and long-term antimicrobial bone concentrations without reaching toxic serum levels, several commercial local delivery systems are available for infectious orthopedic surgery (22). The present study reports the use of a single gentamicin dose. However, a repeated exposure resulting in a long-term steady concentration might have reduced the observed effective concentrations. Thus, the present study cannot provide specific clinical recommendations about aminoglycoside doses for local administration, although the first dose of an aminoglycoside is the most important in the course of therapy due to adaptive bacterial resistance (20). Recently, a clinical study demonstrated that debridement and long-term local gentamicin (17.5 mg/ml) released from a gentamicin-loaded calcium sulfate-hydroxyapatite biocomposite were very effective for treating osteomyelitis (23). Gentamicin-loaded polymethyl methacrylate (PMMA) beads (5.4 mg gentamicin per bead with 10, 30, or 60 beads in a chain) are a commonly used local antimicrobial delivery system in orthopedics, although with infection, recurrence rates are commonly reported to be above 10% and are sometimes as high as 45% (23, 24). The present study supports the new approach of an increased focus on local antibiotic treatment of osteomyelitis applied during surgery (23). However, our results indicate that the effective doses of locally administered antimicrobials might be higher than assumed. Thus, the present study should encourage infectious disease specialists and microbiologists to focus on the fact that the *in vivo* susceptibility and pharmacokinetics (including the diffusion distance) of locally administered antimicrobials within implanted and infected tissue are very limited.

Adding antimicrobials to the inoculum of animal models of infectious disease can represent a new proof of concept for *in vivo* single-dose susceptibility testing. Preinoculation mixing of the inoculum with antimicrobials secures an optimal and reproducible contact between the bacteria and the drug within the tissue. Based on this method, the killing of bacteria is based on exposure to a single dose of local antimicrobials under the influence of *in vivo*-specific factors, like inflammation. This approach to antimicrobial susceptibility testing has, to our knowledge, not previously been used.

MATERIALS AND METHODS

Bacterial strain used in the porcine model. A porcine biofilm-forming *S. aureus* strain, S54F9 *spa* type t1333, was used in the present study (25, 26). The presence of genes coding for a number of toxins, such as enterotoxins, including phage-associated enterotoxins, exotoxins, and superantigen, in this strain has previously been demonstrated by whole-genome sequencing (25).

MIC of gentamicin. The MIC value for *S. aureus* S54F9 was determined in accordance with the guidelines from the Clinical and Laboratory Standards Institute (CLSI) (27). The bacterial strain was inoculated in Luria-Bertani (LB) broth for 24 h at 37°C. Thereafter, 10 μl of 10-fold dilutions was inoculated onto blood agar plates, and the plates were incubated overnight at 37°C in order to

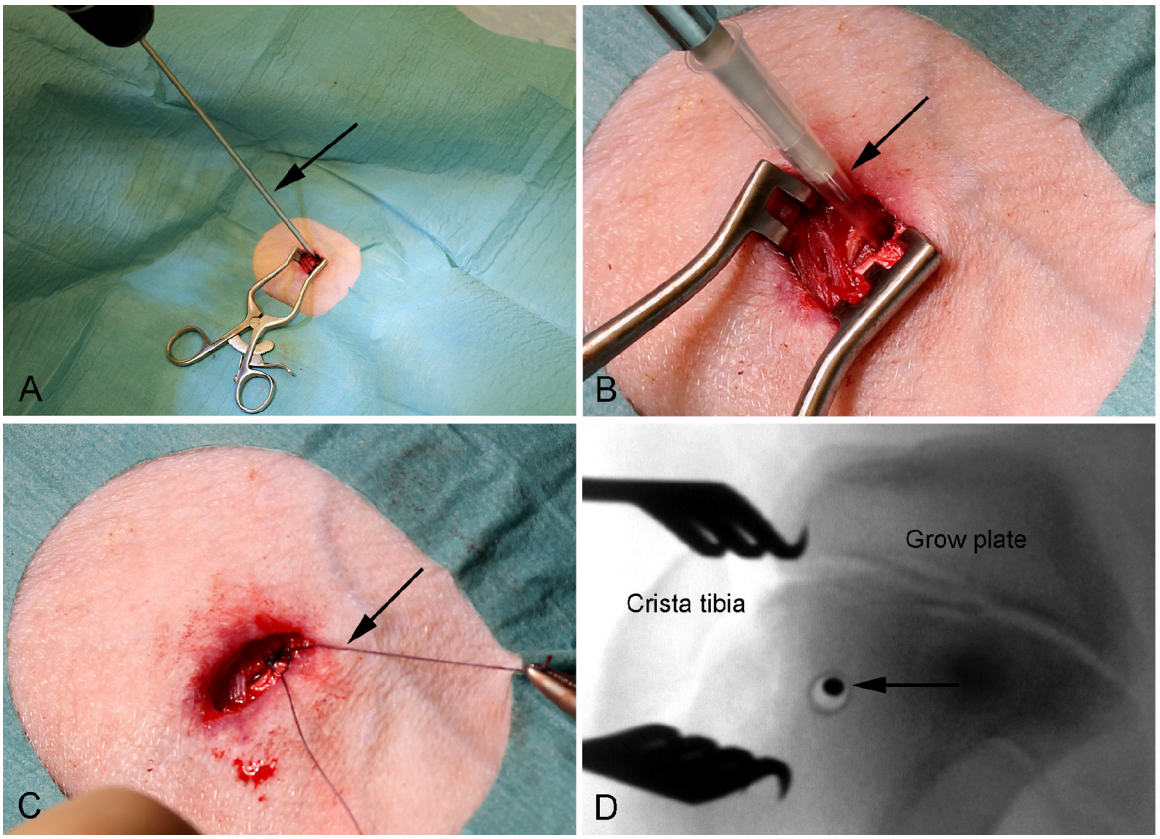

FIG 5 Inoculation procedure in a porcine model of implant-associated osteomyelitis. (A) A small incision down to the periosteum was made on the medial side of the proximal right tibia, and a 4-mm K wire (arrow) was drilled 2 cm into the bone to establish the implant cavity. (B) The inoculum containing *S. aureus* bacteria and different concentrations of gentamicin (arrow) was injected into the implant cavity. (C) After inoculation, a steel implant of 2 by 15 mm (made from a K wire) was inserted into the implant cavity, and the periosteum was closed with sutures (arrow), followed by closure of the soft tissue and skin. (D) Fluoroscopy just after the operation. The implant (arrow) is seen in the implant cavity.

determine the exact bacterial concentration. Sterile isotonic saline (0.9%) was used to achieve a final concentration of 5×10^5 CFU/ml to be used in each test tube (1 ml). One milliliter of gentamicin (100 mg/ml; Genta-Equine; Dechra, Lostock Gralam, UK) at different concentrations (8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, and 0.03 mg/liter) was also added to the test tube. Positive- and negative-control tubes were also included. The MIC value was determined from the tube with the highest dilution of gentamicin with no visible bacterial growth after 24 h at 37°C.

Time-kill curve study of gentamicin. Five different concentrations of gentamicin were used, based on the previous MIC determinations, i.e., 16, 160, 1,600, 16,000, and 160,000 times the MIC value. *Staphylococcus aureus* S54F9 was inoculated in LB broth for 24 h at 37°C. The bacterial suspension was diluted in Mueller-Hinton (MH) bouillon to reach a final concentration of 10^7 CFU/ml. Of this suspension, 15 ml was transferred to each of six sterile Erlenmeyer flasks, and 1 ml of gentamicin at one of the different concentrations was added. One control without gentamicin was also included. Samples of 0.5 ml were taken at the baseline (−5 min, control flasks only), at 1 min after gentamicin exposure, and again after 1, 2, 4, 6, and 24 h. The samples were centrifuged at 15,000 × g for 2 min and resuspended in 1.5 ml sterile isotonic saline (0.9%) in order to minimize the gentamicin carryover effect. This procedure was repeated 4 times. The last time, the pellet was resuspended in the original volume of 0.5 ml. Thereafter, 10-fold dilutions of the samples were made, 10 μl of each dilution was spread out on blood agar plates, and the plates were incubated for approximately 24 h at 37°C. Afterwards, viable counts were performed. The detection limit was 10^2 CFU/ml.

Experimental animals. Twenty-five pigs (female, Danish Landrace) obtained from specific-pathogen-free herds were included. The pigs were 2 to 3 months old and weighed 30 to 40 kg. Some data from 12 of the pigs (groups A and B) (Table 1), which were the basis for the development of the porcine model of osteomyelitis, were recently published (13).

Animal experiment. A skin incision down to the periosteum was made over the final position for the implant cavity, i.e., 10 mm distal to and parallel with the growth plate of the proximal tibia (13). A final incision of 10 mm was made in the periosteum, which was loosened a few millimeters perpendicular to the incision. In the periosteal incision, a K wire (4 mm in diameter) was drilled 20 mm into the trabecular bone tissue, creating the implant cavity (Fig. 5A). The inoculum (10 μl) was prepared (see "Inoculum"

below) and injected (Fig. 5B) into the implant cavity. Afterwards, the implant (steel K wire of 2 by 15 mm) was inserted in the cavity and the periosteum, soft tissue, and skin were closed (Fig. 5C and D) (13). See Table 1 for the different groups based on the different inocula. The pigs were euthanized after 5 days by an intravenous overdose of pentobarbital (20%). All animals received daily oral analgesic treatment with meloxicam (0.3 mg/kg of body weight; Metacam). The Danish Animal Experiments Inspectorate approved the experimental protocol (license no. 2013/15-2934-00946).

Inoculum. Group A animals were inoculated with sterile saline. Pigs in group B were inoculated with *S. aureus* S54F9 *at* 10^4 CFU in 10 μl (25, 28). Pigs in groups C, D, E, and F were given gentamicin (100 μg/ml; Genta-Equine) at different concentrations along with the bacteria. Ten microliters with double gentamicin concentration (2×160, 1,600, 16,000, or 160,000 times the MIC) and 10 μl of 2×10^4 CFU were mixed for 60 s before 10 μl of this solution was inoculated into the tibial implant cavity.

Microbiology and sonication of implants. Following euthanasia, the bone implants were analyzed for biofilm attachment. The implants were placed in centrifuge tubes containing 2 ml 0.9% NaCl. The implants were placed in an ultrasound bath (Bransonic model 2510 bath; Branson Ultrasonic Corporation), degassed for 5 min, sonicated for 5 min, and subsequently vortexed, serially diluted, and plated on blue agar plates (29). The plates were incubated at room temperature for 2 days before determination of the number of CFU per milliliter (29). Following removal of the implants, a swab specimen of the implant cavity was taken and spread over LB agar medium, and the plate was incubated for 24 h at 37°C. Afterwards, the swab isolates were *spa* typed (30). Evaluation of swabs, *spa* typing, and sonication results were performed in a blind manner.

Pathology. The right inoculated tibial bones were sectioned sagittally through the implant cavity in order to allow macroscopic evaluation. Afterwards, the tibial bones were fixed and decalcified (13). Tissue blocks containing the implant cavity and the surrounding bone tissue were embedded in paraffin wax (13). Tissue sections (4 to 5 μm) were stained with hematoxylin and eosin (HE). On a tissue section containing the center of the implant cavity, the size of the peri-implant pathological bone area (PIBA) was measured perpendicular to the implant cavity (13). Within PIBA, biofilm aggregates were detected based on immunohistochemical (IHC) staining of *S. aureus* (28), and the number of neutrophilic granulocytes (NG) was counted by the method developed by Morawietz et al. (31). First, potential hot spots rich in NG were identified. These areas were then evaluated under high power (×400 magnification), and all cells identifiable as NG were counted. A maximum of 10 NG was counted in 10 high-power fields, resulting in a maximum count per pig of 100 NG (31). All PIBA measurements, counting of bacterial aggregates, and NG counts were obtained in a blind manner.

Statistics. By adding the data for groups C and D (bacteria plus 160× and 1,600× MIC of gentamicin, respectively) and those for groups E and F (bacteria plus 16,000× and 160,000× MIC of gentamicin, respectively), a low-MIC-value group (<10,000× MIC) and a high-MIC-value group (>10,000× MIC), respectively, were established. The Kruskal-Wallis test followed by Dunn's multiple-comparison test were used to analyze the number of bacteria attached to the implants between group B (bacteria only) and the low- and high-MIC-value groups. A *P* value below 0.05 was considered significant.

ACKNOWLEDGMENTS

We thank Betina Andersen and Elizabeth Petersen for excellent laboratory assistance.

Louise Kruse Jensen, Bent Aalbæk, Nicole Lind Henriksen, and Henrik Elvang Jensen designed the study. Louise Kruse Jensen and Nicole Lind Henriksen performed the surgical insertion of bone implants. Louise Kruse Jensen and Nicole Lind Henriksen performed the necropsies. Bent Aalbæk, Thomas Bjarnsholt, Kasper N. Kragh, Andreas Petersen, Karen Pankoke, and Sophie Amalie Blirup performed the microbiology, e.g., preparation of the inoculum, screening for bacteremia, sonication, swab analysis, and *spa* typing. Bent Aalbæk, Sophie Amalie Blirup, and Karen Pankoke performed the MIC calculations and time-kill study. Louise Kruse Jensen, Henrik Elvang Jensen, Sophie Amalie Blirup, and Karen Pankoke performed the histopathology. Louise Kruse Jensen drafted the manuscript. All authors read, revised, and approved the final submitted manuscript.

We declare no financial or personal conflict of interest.

This study was financed by grant no. 4005-00035B from the Danish Research Council, the European Union's Horizon 2020 research and innovation program under NOMORFILM project grant agreement no. 634588, and grant no. R173-2014-584 and R105-A9791 from the Lundbeck Foundation to T.B.

REFERENCES

1. Bjarnsholt T. 2013. The role of bacterial biofilm in chronic infections. APMIS Suppl 121:1–58. https://doi.org/10.1111/apm.12099.
2. Maciá MD, Rojo-Molinero E, Oliver A. 2014. Antimicrobial susceptibility testing in biofilm-growing bacteria. Clin Microbiol Infect 20:981–990. https://doi.org/10.1111/1469-0691.12651.
3. Moskowitz SM, Foster JM, Emerson J, Burns JL. 2004. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J Clin Microbiol 5:1915–1922. https://doi.org/10.1128/JCM.42.5.1915-1922.2004.
4. Riera E, Maciá MD, Mena A, Mulet X, Pérez JL, Ge Y, Oliver A. 2010.

Anti-biofilm and resistance suppression activities of CXA-101 against chronic respiratory infection phenotypes of *Pseudomonas aeruginosa* strain PAO1. J Antimicrob Chemother 65:1399–1404. https://doi.org/10.1093/jac/dkq143.
5. Fernández-Olmos A, García-Castillo M, Maiz L, Lamas A, Baquero F, Cantón R. 2012. *In vitro* prevention of *Pseudomonas aeruginosa* early biofilm formation with antibiotics used in cystic fibrosis patients. Int J Antimicrob Agents 40:173–176. https://doi.org/10.1016/j.ijantimicag.2012.04.006.
6. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. 1999. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol 37:1771–1776.
7. Stewart PS. 2015. Animicrobial tolorance in biofilms. Microbiol Spectr 3(3):MB-0010-2014. https://doi.org/10.1128/microbiolspec.MB-0010-2014.
8. Wu H, Moser C, Wang H-Z, Hoiby N, Song Z-J. 2015. Strategies for combating bacterial biofilm infections. Int J Oral Sci 7:1–7. https://doi.org/10.1038/ijos.2014.65.
9. Mottola C, Matias CS, Mendes JJ, Melo-Cristino J, Tavares L, Cavaco-Silva P, Oliveira M. 2016. Susceptibility patterns of Staphylococcus aureus biofilms in diabetic foot infections. BMC Microbiol 16:119. https://doi.org/10.1186/s12866-016-0737-0.
10. Howlin RP, Brayford MJ, Webb JS, Cooper JJ, Aiken SS, Stoodley P. 2015. Antibiotic-loaded synthetic calcium sulfate beads for prevention of bacterial colonization and biofilm formation in periprosthetic infections. Antimicrob Agents Chemother 59:111–120. https://doi.org/10.1128/AAC.03676-14.
11. Coenye T, Goeres D, Van Bambeke F, Bjarnsholt T. 2018. Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? Clin Microbiol Infect 24:570–572. https://doi.org/10.1016/j.cmi.2018.01.003.
12. Jensen LK, Koch J, Henriksen NL, Bue M, Tøttrup M, Hanberg P, Søballe K, Jensen HE. 2017. Suppurative inflammation and local tissue destruction reduce the penetration of cefuroxime to infected bone implant cavities. J Comp Pathol 4:308–316. https://doi.org/10.1016/j.jcpa.2017.10.001.
13. Jensen LK, Koch J, Dich-Jorgensen K, Aalbaek B, Petersen A, Fuursted K, Bjarnsholt T, Kragh KN, Tøtterup M, Bue M, Hanberg P, Søballe K, Heegaard PMH, Jensen HE. 2017. Novel porcine model of implant-associated osteomyelitis: a comprehensive analysis of local, regional and systemic response. J Orthop Res 35:2211–2221. https://doi.org/10.1002/jor.23505.
14. Butini ME, Cabric S, Trampuz A, Di Luca M. 2018. *In vitro* anti-biofilm activity of a biphasic gentamicin-loaded calcium sulfate/hydroxyapatite bone graft substitute. Colloids Surf B Biointerfaces 161:252–260. https://doi.org/10.1016/j.colsurfb.2017.10.050.
15. Bjarnsholt T, Alhede M, Alhede M, Eickhardt-Sørensen SR, Moser C, Kühl M, Jensen PØ, Høiby N. 2013. The in vivo biofilm. Trends Microbiol 21:466–474. https://doi.org/10.1016/j.tim.2013.06.002.
16. Ackermann MR. 2017. Inflammation and healing, p 73–131. *In* Zachary JF (ed). Pathologic basis of veterinary disease, 6th ed. Mosby Elsevier, St. Louis, MO.
17. McCourt J, O'Halloran DP, McCarthy H, O'Gara JP, Geoghegan JA. 2014. Fibronectin-binding proteins are required for biofilm formation by community-associated methicillin-resistant *Staphylococcus aureus* strain LAC. FEMS Microbiol Lett 353:157–164. https://doi.org/10.1111/1574-6968.12424.
18. Kwiecinski J, Kahlmeter G, Jin T. 2015. Biofilm formation by *Staphylococcus aureus* isolates from skin and soft tissue infections. Curr Microbiol 70:698–703. https://doi.org/10.1007/s00284-014-0770-x.
19. Baudoux P, Bles N, Lemaire S, Mingeot-Leclercq M-P, Tulkens PM, van Bambeke F. 2006. Combined effect of pH and concentration on the activities of gentamicin and oxacillin against *Staphylococcus aureus* in pharmacodynamic models of extracellular and intracelullar infections. J Antimicrob Chemother 59:246–253. https://doi.org/10.1093/jac/dkl489.
20. Tam VH, Kabbara S, Vo G, Schilling AN, Coyle EA. 2006. Comparative pharmacodynamics of gentamicin against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 50:2626–2631. https://doi.org/10.1128/AAC.01165-05.
21. Ruppen C, Hemphill A, Sendi P. 2017. In-vitro activity of gentamicin as adjunct to penicillin against biofilm group B Streptococcus. J Antimicrob Chemother 72:444–447. https://doi.org/10.1093/jac/dkw447.
22. Diefenbeck M, Mückley T, Hofmann GO. 2006. Prophylaxis and treatment of implant-related infections by local application of antibiotics. Injury 37(Suppl 2):S95–S104. https://doi.org/10.1016/j.injury.2006.04.015.
23. McNally M, Ferguson JY, Lau ACK, Diefenbeck M, Scarborough M, Ramsden AJ, Atkins BL. 2016. Single-stage treatment of chronic osteomyelitis with a new absorbable gentamicin-loaded, calcium sulphate/hydroxyapatite biocopostite. Bone Joint J 98-B:1289–1296. https://doi.org/10.1302/0301-620X.98B9.38057.
24. Klemm K. 2001. The use of antibiotic-coating bead chains in the treatment of chronic bone infections. Clin Microbiol Infect 7:28–31. https://doi.org/10.1046/j.1469-0691.2001.00186.x.
25. Aalbæk B, Jensen LK, Jensen HE, Olsen JE, Christensen H. 2015. Whole-genome sequence of *Staphylococcus aureus* S54F9 isolated from a chronic disseminated porcine lung abscess and used in human infection models. Genome Announc 3(5):e01207-15. https://doi.org/10.1128/genomeA.01207-15.
26. Hasman H, Moodley A, Guardabassi L, Stegger M, Skov RL, Aarestrup FM. 2010. Spa type distribution in *Staphylococcus aureus* originating from pigs, cattle and poultry. Vet Microbiol 141:326–331. https://doi.org/10.1016/j.vetmic.2009.09.025.
27. Watts JL, Shryock TR, Apley M, Bade DJ, Brown SD, Gray JT, Heine H, Hunter RP, Mevius DJ, Papich MG, Silley P, Zurenko GE. 2008. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard, 3rd ed, p 31–35. CLSI document M31-A3. Clinical and Laboratory Standards Institute, Wayne, PA.
28. Johansen LK, Frees D, Aalbaek B, Koch J, Iburg T, Nielsen OL, Leifsson PS, Jensen HE. 2011. A porcine model of acute haematogenous localized osteomyelitis due to *Staphylococcus aureus*: a pathomorphological study. APMIS 119:111–118. https://doi.org/10.1111/j.1600-0463.2010.02700.x.
29. van Gennip M, Christensen LD, Alhede M, Qvortrup K, Jensen PØ, Høiby N, Givskov M, Bjarnsholt T. 2012. Interactions between polymorphonuclear leukocytes and *Pseudomonas aeroginosa* biofilms on silicone implants *in vivo*. Infect Immun 80:2601–2607. https://doi.org/10.1128/IAI.06215-11.
30. Stegger M, Andersen PS, Kearns A, Pichon B, Holmes MA, Edwards G, Laurent F, Teale C, Skov R, Larsen AR. 2012. Rapid detection, differentiation and typing of methicillin-resistant Staphylococcus aureus harbouring either mecA or the new mecA homologue mecA(LGA251). Clin Microbiol Infect 18:395–400. https://doi.org/10.1111/j.1469-0691.2011.03715.x.
31. Morawietz L, Tiddens O, Mueller M, Tohtz S, Gansukh T, Schroeder JH, Perka C, Krenn V. 2009. Twenty-three neutrophil granulocytes in 10 high-power fields is the best histopathological threshold to differentiate between aseptic and septic endoprosthesis loosening. Histopathology 54:847–853. https://doi.org/10.1111/j.1365-2559.2009.03313.x.